**Title:** METHOD OF PREPARING A YEAST-CELL LYTIC ENZYME SYSTEM

**Abstract**

Continuous method for preparing a yeast cell lytic enzyme system having desired beta (1→3) glucanase and protease activities. A suitable bacterium which produces the lytic enzyme system is inoculated onto an appropriate growth medium containing predetermined amounts of a carbon source (for example, sugar) and an inducer of lytic enzyme activity. The bacterium is continuously fermented aerobically, the dilution rate being varied to obtain the desired beta (1→3) glucanase and protease activities. The bacterium may be one or more of the group consisting of *Cytophaga*, *Oerskovia* or *Arthrobacter*. 
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METHOD OF PREPARING A
YEAST-CELL Lytic ENZYME SYSTEM

Background of the Invention

This invention is a method for preparing a yeast cell
lytic enzyme system having desired beta (1→3) glucanase
and protease activities. The method provides an effi-
cient and continuous way to synthesize a lytic enzyme
system.

Yeast cell walls consist of two layers. The outer
layer is a mannan-protein complex and the inner layer
is an alkali-insoluble glucan. To break the wall the
presence of both glucanase and protease enzymes are
necessary. Only small amounts of protease seem to be
required. To break brewe'r's yeast cells, glucanase
displays more profound lytic action than the lytic
enzyme (glucanase and protease) alone after a brief
treatment of the cells with lytic enzyme. The protease
appears to be important in the initial attack on the
cell surface. The opening of polypeptide chains by the
protease makes the inner glucan layer of the wall ac-
cessible to the glucanase.

Yeast cell lytic enzyme systems may be produced by
either batch or continuous fermentation. The continu-
ous process is superior to the batch process with re-
gard to enzyme production. Furthermore, the composi-
tion (constituent enzymes) of the lytic enzyme systems
can be manipulated by the use of different inducers and
by altering the dilution rate. The existence of lytic
enzyme systems with different component activities has
a potential for their use in specialized applications.
Such applications include production of food grade
protein and intracellular enzymes, isolation of
intracellular recombinant protein produced in yeast, digestion of cell wall polysaccharides, production of protoplasts, as anticaries agents, in the study of cell wall structure, in the treatment of fungal diseases and as an essential tool for cell fusion, transformation and genetic engineering of yeast.

GB 1,048,887 discloses a process for producing an enzyme complex having strong proteolytic activity. The organism used to produce this enzyme complex is a species of the genus *Cytophaga*. Media compositions and conditions are described for culturing *Cytophaga* NCIB 9497 in a batch system. Centrifugation of the crude product of the culture and precipitation from aqueous solution with a protein precipitant are described as methods of recovery.

GB 1,179,935 discloses a process for producing cytolytic enzymes which are capable of lysing the living cells of microorganisms. The system is a batch fermentation of *Cytophaga johnsonii* as well as other species of the genus *Cytophaga*.

GB 1,186,998 discloses another process for producing cytolytic enzymes of microorganisms in a batch fermentation. The presence of cells of other microorganisms or decomposes thereof in the nutrient medium results in accelerated production of the cytolytic enzymes. *Cytophaga johnsonii* as well as other *Cytophaga* may be cultured under the disclosed invention.

US 3,716,452 discloses an enzyme for lysing yeast cell walls. The enzyme is produced in a batch fermentation of microorganisms belonging to *Arthrobacter luteus* nov. sp. The enzyme has activity for lysing cell walls of yeast dead or alive and at any stage of growth.
Lilley, et al. (J. appl. Chem. Biotechnology, vol. 24, pp. 677-686, (1974)) describe continuous-flow cultivation of a thermophilic streptomycete in a medium containing glycerol as the principal carbon source. Levels of beta (1→3) glucanase in the fermentation broth increased sharply as the dilution rate was decreased below 0.20 h⁻¹.

Obata, et al. (J. Ferment. Technol., vol. 53, no. 5, pp. 256-263, (1975)) describe culture conditions for batch production of yeast lytic enzymes from Gerskoviya sp. ck. The culture filtrate exhibited high lytic activity toward cells of many species of yeast when the yeast cells were used as substrate. In particular, it was found that glucan, the main component of yeast cell wall, and laminarin (which has a structure similar to glucan) were effective inducers. Beta (1→3) glucanase and protease were two of several enzymes produced.

Rowley and Bull (Biotechnology and Bioengineering, vol. 19, pp. 879-899 (1977)) describe the production of lytic enzyme complex in batch and continuous-flow fermenters using a yeast-lyzing Arthrobacter species. In growth medium containing whole yeast cells as inducers, an extracellular lytic enzyme complex was produced which included beta (1→3) glucanase and protease. Optimum activity of these two enzymes was achieved at a dilution rate of 0.05 h⁻¹.

The potential of continuous-flow enrichment culture for the selection of microorganisms producing extra cellular enzymes has been little realized. Continuous culture has a big advantage over batch culture in relation to enzyme production. In continuous culture studies it
has been possible to increase the concentration of the lytic enzymes by more than one order of magnitude compared to the batch production. It is also possible to regulate the ratio of glucanase to protease in the lytic enzyme system by using different inducers or altering the dilution rate.

This study has shown that in continuous culture it is possible to produce enzyme systems with desired characteristics for a particular application (e.g. for wall degradation or to isolate a particular product from inside the cell).
Summary of the Invention

The present invention is a method for preparing a yeast cell lytic enzyme system having desired beta (1→3) glucanase and protease activities. A suitable bacterium which produces the lytic enzyme system is inoculated onto an appropriate growth medium containing predetermined amounts of a sugar as the carbon source (for example, sugar) and an inducer of lytic enzyme activity. The bacterium is continuously fermented aerobically at an appropriate dilution rate, the dilution rate being varied to obtain the desired beta (1→3) glucanase and protease activities. The resulting lytic enzyme system is recovered.

The bacterium may be one or more of the group consisting of *Cytophaga*, *Oerskovia* or *Arthrobacter*. 
Description of the Figures

Fig. 1  Cell and Enzyme Concentrations in *Cytophaga* NCIB 9497 Continuous Culture as a function of dilution rate (h⁻¹). The medium is comprised of 5 g/L glucose and 10 g/L yeast extract. Values are for beta (1→3) glucanase (B(1→3), U/L, (▲)); reducing sugars (R.S., g/L, (●)); protease (P, u, (□)) (u=1% hydrolysis in 20 min.); yeast lytic activity (YLA, U, (△)) (U = % decrease in OD₆₇₀ after 15 min.); Cells (Cells, OD ₆₇₀, (O)).

Fig. 2  Specific Enzyme Activity in Continuous Culture of *Cytophaga* sp. (activity/g cells) as a function of dilution rate (h⁻¹). The medium is comprised of 5 g/L glucose and 10 g/L yeast extract. Values are for yeast lytic activity (YLA, % decrease in OD₆₇₀ after 15 min./g cells, (△)); protease (P, u/g cells, (□)); beta (1→3) glucanase (B(1→3), U/L/g cells, (▲)).

Fig. 3.  Specific Rate of Enzyme Synthesis in Continuous Culture of *Cytophaga* sp. (activity/g cells h). The medium is comprised of 5 g/L glucose and 10 g/L yeast extract. Values are for beta (1→3) glucanase (B(1→3), U/L/g cells h, (▲)); protease (P, u/g cells h, (□)); yeast lytic activity (YLA, % decrease in OD/g cells h, (△)).

Fig. 4  Productivity in Continuous Culture of *Cytophaga* sp. (activity/L h). The medium is comprised of 5 g/L glucose and 10 g/L yeast extract. Values are for yeast lytic activity (YLA, % decrease in OD/L h, (△)); protease (P, u/L h, (□)); beta (1→3) glucanase (B(1→3), U/L/L h, (▲)); Cells (Cells, OD₆₇₀/L h, (O)).
Fig. 5 Cell and Enzyme Concentrations in *Oerskovia xanthineolytica* Continuous Culture as a function of dilution rate (h$^{-1}$). The medium is comprised of 2 g/L glucose and 2 g/L glucan. Values are for beta (1→3) glucanase (B(1→3), U/L, (▲)); protease (P, u, (□)); reducing sugars (R.S., g/L, (●)); yeast lytic activity (YLA, U, (Δ)) (U = % decrease in OD$_{670}$ in 15 min.); Cells (Cells, (#/mL) x 10$^9$, (〇)); mannanase (M, U/L, (〇)).

Fig. 6 Cell and Enzyme Concentration in *Oerskovia xanthineolytica* Continuous Culture as a function of dilution rate (h$^{-1}$). The medium is comprised of 8 g/L glucose and 0.5 g/L glucan. Values are for beta (1→3) glucanase (B(1→3), U/L, (▲)); protease (P, u, (□)); glucose (G, g/L, (●)); yeast lytic activity (YLA, U, (Δ)) (U = % decrease in OD$_{670}$ in 15 min.); Cells (Cells, (#/mL) x 10$^9$, (〇)).

Fig. 7 Beta (1→3) Glucanase and Protease Activities in Continuous Culture of *Oerskovia* sp. as a function of dilution rate (h$^{-1}$). The medium is comprised of 8 g/L glucose and 0.5 g/L glucan or 2 g/L glucose and 2 g/L glucan. Values are for beta (1→3) glucanase (B(1→3), U/L, (▲)); protease (P, u, (□)).

Fig. 8 Specific Enzyme Activity in Continuous Culture of *Oerskovia* sp. (activity/g cells) as a function of dilution rate (h$^{-1}$). The medium is comprised of 2 g/L glucose and 2 g/L glucan. Values are for beta (1→3) glucanase (B(1→3), U/L/g cells, (▲)); protease (P, u/g cells, (□)); mannanase (M, U/L/g cells, (〇)); yeast lytic activity (YLA, % decrease in OD/g cells, (Δ)).
Fig. 9 Specific Enzyme Activity in Continuous Culture of *Oerskovia* sp. (activity/g cells) as a function of dilution rate (h⁻¹). The medium is comprised of 8 g/L glucose and 0.5 g/L glucan. Values are for beta (1→3) glucanase (B(1→3), U/L/g cells, (▲)); protease (P, u/g cells, (□)); yeast lytic activity (YLA, % decrease in OD/g cells, (△)).

Fig. 10. Specific Rate of Enzyme Synthesis in Continuous Culture of *Oerskovia* sp. (activity/g cells h) as a function of dilution rate (h⁻¹). The medium is comprised of 2 g/L glucose and 2 g/L glucan. Values are for protease (P, u/g cells h, (□)); mannanase (M, U/L/g cells h, (○)); beta (1→3) glucanase (B(1→3), U/L/g cells h, (▲)); yeast lytic activity (YLA, % decrease in OD/g cells h, (△)).

Fig. 11 Specific Rate of Enzyme Synthesis in Continuous Culture of *Oerskovia* sp. (activity/g cells h) as a function of dilution rate (h⁻¹). The medium is comprised of 8 g/L glucose and 0.5 g/L glucan. Values are for protease (P, u/g cells h, (□)); beta (1→3) glucanase (B(1→3), U/L/g cells h, (▲)); yeast lytic activity (YLA, % decrease in OD/g cells h, (△)).

Fig. 12 Productivity in Continuous Culture of *Oerskovia* sp. as function of dilution rate (h⁻¹). The medium used was comprised of 2 g/L glucose and 2 g/L glucan. Values are for yeast lytic activity (YLA, % decrease in OD/L h, (△)); beta (1→3) glucanase (B(1→3), U/L/L h, (▲)); protease (P, u/L h, (□)); mannanase (M, U/L/L h, (○)); Cells (Cells, (#/mL) x 10⁹, (○)).
Fig. 13 Productivity in Continuous Culture of Ceratoxia sp. as a function of dilution rate (h⁻¹).

The medium used was comprised of 8 g/L glucose and 0.5 g/L glucan. Values are for yeast lytic activity (YLA, % decrease in OD/L h, (△)); beta (1→3) glucanase (B1→3), U/L/L h, (▲)); protease (P, u/L h, (□)); dry weight (D.W., g/L/L h (O)).
Detailed Description of the Invention

The present invention is a method for preparing a yeast cell lytic enzyme system having desired beta (1→3) glucanase and protease activities. A suitable bacterium which produces the lytic enzyme system is inoculated onto an appropriate growth medium containing predetermined amounts of carbon source and an inducer of lytic enzyme activity. The bacterium is continuously fermented aerobically at an appropriate dilution rate, the dilution rate being varied to obtain the desired protease and beta (1→3) glucanase activities. The resulting lytic enzyme system is recovered.

The bacterium may be one or more of the group consisting of Cytophaga, Oerskonia or Arthrobacter.

The method may consist of inoculating Oerskonia onto a growth medium containing glucose as the carbon source and yeast wall glucan as the inducer. The growth medium may contain up to 20 g/L of glucose and 20 g/L of yeast wall glucan. In particular, the growth medium may contain 8 g/L glucose and 0.5 g/L yeast wall glucan. The dilution rate for the continuous fermentation may be selected so it provides high beta (1→3) glucanase activity. In particular, the dilution rate may be between about 0.05 and about 0.20 h⁻¹ for high beta (1→3) glucanase activity. The dilution rate may also be selected so it provides high protease activity. In particular, the dilution rate may be between about 0.02 and about 0.10 h⁻¹ for high protease activity. The dilution rate may be selected so it provides high beta (1→3) glucanase activity and low protease activity. The dilution rate may be between about 0.15 and about 0.20 h⁻¹ for high beta (1→3) glucanase activity and low protease activity.
Another growth medium for *Oerskonia* may contain 2 g/L glucose as the carbon source and 2 g/L yeast wall glucan as the inducer. The dilution rate for the continuous fermentation may be selected so it provides high beta (1→3) glucanase activity. In particular, the dilution rate may be between about 0.05 and about 0.14 h⁻¹ for high beta (1→3) glucanase activity. The dilution rate may also be selected so it provides high protease activity. In particular, the dilution rate may be between about 0.02 and about 0.14 h⁻¹ for high protease activity. The dilution rate may also be selected so it provides high beta (1→3) glucanase and low protease activity. In particular, the dilution rate may be between about 0.15 and about 0.20 h⁻¹ for high beta (1→3) glucanase activity and low protease activity.

The amount of inducer present may be an amount sufficient to produce high levels of beta (1→3) glucanase activity.

Bacterial strains suitable for the method of this invention include wild type strains of *Cytophaga*, *Oerskonia* and *Arthrobacter* and derivatives thereof.

The dilution rate which is suitable for the present invention may be between about 0.02 and about 0.40 h⁻¹.

The continuous culture may be maintained at a pH and temperature suitable for preparing a yeast cell lytic enzyme system.

Another aspect of the invention may consist of inoculating *Cytophaga* onto a growth medium containing glu-
cose as a carbon source. In particular, the growth medium may contain 5 g/L glucose. The dilution rate for the continuous fermentation may be selected so it provides high beta (1→3) glucanase activity and high protease activity. The dilution rate may be between about 0.05 and about 0.10 h⁻¹ for high beta (1→3) glucanase and high protease activity. The pH for this method may be about 7.0 and the temperature may be about 29°C.

Synthesis of enzymes may be inducible, semi-constitutive or constitutive. Inducible enzymes are synthesized at a low level in the absence of inducers. The uninduced, basal level of enzyme synthesis can vary considerably. When the basal enzyme activity is high, it is classified as semi-constitutive. Constitutive enzymes are synthesized maximally in the presence or absence of an inducer.

Several bacteria are suitable for the disclosed method. One in particular, *Cytophaga* NCIB 9497, has been considered to be constitutive for yeast lytic enzyme synthesis. When *Cytophaga* NCIB 9497 is inoculated onto a medium of 10 g/L of yeast extract and 5 g/L of glucose, the specific activities of the beta (1→3) glucanase and yeast lytic activity are high at dilution rates between 0.05 and 0.10 h⁻¹. In this range of dilution rates the level of reducing sugars in the fermentation broth is low. Results indicate dual control of beta (1→3) glucanase synthesis by induction and catabolite repression. Between dilution rate of 0.03 and 0.07 h⁻¹ catabolite repression is minimal because the low growth rate of the organism does not lead to an accumulation of repressing catabolites. Synthesis is regulated by the amount of inducer present. Growth
rate increases with increasing dilution rate and as a result the rate of enzyme synthesis increases. At a dilution rate between 0.07 and 0.1 h\(^{-1}\) the concentration of the repressor has reached a level high enough to cause significant catabolite repression. As the dilution rate increases the growth rate increases and catabolite repression becomes dominant over induction and the rate of enzyme production drops.

The regulation of protease enzyme synthesis does not appear to be under dual control by induction and catabolite repression in the range of dilution rates studied (between 0.03 and 0.30h\(^{-1}\)). Protease synthesis is subject to catabolite repression but is not induced.

Reducing sugars in the fermentation broth described above accumulated at dilution rates greater than 0.15 h\(^{-1}\). The reducing sugar content was 6.9 g/L, indicating that most of the measured reducing sugars were glucose. At dilution rates up to 0.3 h\(^{-1}\) washout did not occur. The accumulation of reducing sugars at dilution rates above 0.15 h\(^{-1}\) indicates the presence of a second limiting substrate. This substrate is unknown. Dissolved oxygen was never limiting.

Protease synthesis does not appear, from continuous culture studies, to be inducible. The activity levels therefore are similar in late batch culture and in continuous culture at low dilution rates. Enzyme levels in continuous culture at high dilution rates (when glucose accumulates) are similar to those in batch culture when approximately half the reducing sugars have been metabolized.
It appears that the proteolytic activity is made up of activity from more than one protease enzyme and that the syntheses of these enzymes are not regulated together. In carbon limited media, two peaks of specific rate of enzyme synthesis exist; one at dilution rates above 0.15 h\(^{-1}\) and one at dilution rates below 0.15 h\(^{-1}\). The appreciable rate of enzyme synthesis at high dilution rates is evidence that not all the proteolytic enzymes are catabolite repressed. There is also evidence that one of the proteases may be induced by the second, unknown limiting substrate. The peak at low dilution rates indicates that one of the proteases may be weakly induced.

Another suitable bacterium of the disclosed method is *Oerskovia xanthineolytica*. Continuous cultures were carried out using two different media; one with 2 g/L of glucose and 2 g/L of glucan and the other with 8 g/L of glucose and 0.5 g/L of glucan.

In both continuous cultures enzyme activities were high at low dilution rates (below 0.15 h\(^{-1}\)). The beta (1\(\rightarrow\)3) glucanase activity on the medium with 8 g/L glucose and 0.5 g/L of glucan is higher than that produced on the medium with 2 g/L glucose and 2 g/L glucan. The same is true of protease activity.

There is a distinction between the beta (1\(\rightarrow\)3) glucanase specific activities in the two continuous cultures. In the medium with 2 g/L glucose and glucan there is a sharp peak of beta (1\(\rightarrow\)3) glucanase specific activity at low dilution rates. The maximum specific activity is more than three times that on the medium of 8 g/L glucose and 0.5 g/L of glucan. Beta (1\(\rightarrow\)3) glucanase specific activity in the medium containing 2 g/L glucose
and glucan appears to be controlled by both induction and catabolite repression. Protease does not appear to be induced at low dilution rates, but is subject to catabolite repression.

Materials and Methods

Cytophaga sp. NCIB 9497 was obtained from the National Collection of Industrial Bacteria (Aberdeen, Scotland). Oerskovia xanthineolytica LL-GL09 was a gift from M. Lechavalier, Rutgers University, New Jersey, USA. Arthrobacter sp. GJM-1 was obtained from C. Ballou, University of California, Berkeley, USA. Saccharomyces cerevisiae NCYC 1006 was from the National Collection of Yeast Cultures, Norwich, UK. Saccharomyces carlsbergensis was a gift from the Stroh Brewing Company, Michigan, USA and the bakers yeast used was Red Star active dried yeast from the Universal Food Corporation, Wisconsin, USA.

All chemicals used were Analar grade except those listed below.

Yeast extract - "Ardamine Yep", (autolyzed yeast extract powder), was supplied by Yeast Products Inc., New Jersey, USA.

Glucose (used in fermentation media) - technical grade, Sigma Chemical Company, Missouri, USA.

Nutrient Agar, Maltose and Peptone technical grade - Difco Laboratories, Michigan, USA.
Antifoam - a 30% aqueous solution of a 100% active silicone polymer, Sigma Chemical Company, Missouri, USA.

Glucan, prepared by alkali extraction from bakers yeast, (Manners et al., 1973a) was a gift from J. Hunter, Department of Chemical Engineering and Applied Chemistry, Columbia University, New York, USA. It was sieved and the fraction smaller than 177 microns (mesh #80) was used.

Yeast cell walls (used as an inducer) were prepared from Saccharomyces carlsbergensis disrupted in a Dyno mill (Willy A. Bachofen AG, Switzerland) for 20 minutes (approximately 90% disruption), washed with distilled water and filtered (Whatman #2 paper).

For the growth of inocula and for some batch experiments Erlenmeyer flasks were used. Cultures were incubated in an incubator-shaker (Model G 26), New Brunswick Scientific Co., Inc., New Jersey, USA. In fermentations with Cytophaga sp. a lyophilised culture was used for each inoculum. Inocula (10% v/v) were grown for 12-17 hours. Bioflo C 30 and C 32 fermenters (New Brunswick Sci. Co. Inc.) were used for both batch and continuous culture studies. The C 32 vessel has a volume of 2 L (1.2 L working volume) and the C 30 a volume of 750 mL (350 mL working volume). These fermenters were fitted with automatic temperature and pH control and a dissolved oxygen (DO) probe and meter.

Media

For Cytophaga sp.:
5.0 g/L glucose
10.0 g/L yeast extract

For *Oerskovia* sp. and *Arthrobacter* sp.:

2.0/8.0 g/L glucose
2.0/0.5 g/L glucan
0.5 g/L K$_2$HPO$_4$
0.3 g/L NaH$_2$PO$_4$·2H$_2$O
5.0 g/L NH$_4$Cl

50 mL Trace element mix

pH 6.8

Trace element mix:

0.250 g/L MgSO$_4$·7H$_2$O
0.050 g/L CaCl$_2$
0.020 g/L ZnSO$_4$·7H$_2$O
0.020 g/L MnSO$_4$·4H$_2$O
0.005 g/L CuSO$_4$·5H$_2$O
0.100 g/L FeSO$_4$·7H$_2$O

1.000 g/L Na$_2$SO$_4$

0.600 g/L EDTA Na$_2$H$_2$O

**ASSAYS**

Cell concentration was estimated in three ways: optical density (OD), dry weight (DW), or viable cell count (colony count).

**Optical Density**

Optical Density was measured in a Spectronic 21 spectrophotometer (Bausch and Lomb, New York, USA). Samples were diluted before reading to fall within the
range from 0 to 0.6 on the absorbance scale. When used to estimate growth optical densities were read at 670 nm.

**Dry Weight**

A known volume of culture was filtered through a previously dried and weighed "Metrice" membrane filter (0.45 microns), Gelman Sciences Inc., Michigan, USA. The filter was then dried to constant weight at 80 C. Dry weight (DW) was calculated and expressed in grams of cells per litre of culture broth.

**Colony Count**

The colony count method was used to estimate growth in fermentations containing insoluble substances where optical density and dry weight were not useful. Serial dilutions of the culture broth were made in sterile distilled water. 0.1mL of the appropriate dilutions were plated in duplicate. After 72 hours the colonies were counted and the number of cells per unit volume calculated.

Continuous fermentations were carried out in Bioflo C 30 or C 32 fermenters. 3 volume changes were allowed before steady state samples were taken and 3 samples were taken at 1 hour intervals at each point. The fraction of the original material remaining in the chemostat after 3 volume changes is 0.05 (Pirt, 1975). When using the 350mL (C 30) fermenter the smallest possible samples (less than 5 mL) were taken to avoid altering the fermenter volume and thus the steady state conditions more than necessary.
Sterile antifoam was added aseptically to the medium reservoir after sterilisation at a concentration of 0.25 mL/L of a 30% aqueous solution per litre of medium. If added before autoclaving clumps formed on the surface of the medium and the effect of the antifoam was lost, the performance of the DO probe was also affected.

When insoluble inducers were used they were added directly to the medium reservoir which was stirred throughout the fermentation. The tubing (silicone) was checked daily for buildups of insoluble material.

Culture purity was checked daily by plating two 0.1 mL samples of culture broth on nutrient agar plates.

Immediately after removal from the fermenter the sample to be used for enzyme assays was filtered (membrane filter, 0.45 microns) and frozen. When larger volumes of enzyme were needed the supernatent was centrifuged at 2000 rpm for 15 minutes, separated and the enzyme preparation frozen.

**Yeast Lytic Activity**

The method used to estimate the overall lytic activity was decrease in optical density in an incubation of whole yeast cells, lytic enzyme and buffer.

Decrease in optical density: equal volumes of enzyme preparation, yeast substrate (a 1/400 w/v solution of washed, packed cells) and buffer (50 mM Tris-HCl, pH 7.3) were mixed in a spectrophotometer tube which was then incubated at 37 C. Periodically the tube was removed from the water bath, mixed and the optical density at 670 nm read. Yeast autolysis was followed
by incubating 1 volume of yeast with 2 volumes of buffer. Yeast lytic activity is expressed in units (U) where 1U equals a 1% decrease in OD after 15 min. in the range of 20% to 50% decrease.

Glucanase and Mannanase Activities

Beta (1→3) Glucanase activity assay: equal volumes of enzyme, laminarin (a 3% w/v solution) and buffer (50 mM Tris-HCl, pH 7.3) were incubated at 37 C. The reaction was terminated by boiling in a water bath for 5 minutes. Reducing sugars released were assayed using the Nelson-Somogyi method (Nelson, 1944) or the Micro Nelson method (Spiro, 1966). Standard curves were prepared using glucose.

Mannanase activity was assayed in the same way Beta (1→3) glucanase except that the substrate used was a 0.5% solution of mannose.

Glucanase and mannanase activities are expressed in international units, 1 U is that amount of enzyme which releases 1 umole of glucose per minute under specified conditions.

Proteolytic Activity

Protease activity was measured by the method of Rowley and Bull (1977). The substrate used was azocasein at a concentration of 2.5 g/L. Equal volumes of substrate and enzyme were incubated at 37 C for 20 minutes. The reaction was terminated by the addition of 2 mL of 3 M TCA (trichloroacetic acid). After centrifugation at 2000 rpm for 30 minutes the absorbance of the supernatant was read at 400 nm. Various blanks and controls
were run. The activity is expressed in units (u) where 1 u equals 1% hydrolysis in 20 min. in the range from 0% to 30%. A unit corresponds to the solubilization of 0.00125 mg. protein per min. per ml. of enzyme solution.

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**Glucose Assay**

Glucose concentrations were determined by the PGO (Peroxidase Glucose Oxidase) method, Sigma Technical Bulletin No. 510.

**Example I**

*Cytophaga* NCIB 9497 was grown in continuous culture. The medium contained 10 g/L of yeast extract and 5 g/L of glucose. The pH was controlled at 7 and the temperature at 29°C. Steady state conditions at dilution rates from 0.03 h⁻¹ to 0.30 h⁻¹ were analyzed for cell concentration, beta (1→3) glucanase, protease, mannanase and yeast lytic activities as well as reducing sugars present in the fermentation broth.

The level of glucose (reducing sugars) in the fermentation broth was between 0.5 and 0.8 g/L up to a dilution rate of 0.15 h⁻¹. At dilution rates above 0.15 h⁻¹ glucose accumulated and another substrate became limiting.

At dilution rates above 0.15 h⁻¹ the synthesis of beta (1→3) glucanase is repressed by glucose. Yeast lytic activity is mainly attributable to production of proteolytic enzymes. Activities of beta (1→3) glucanase, protease, mannanase and overall yeast lytic activity are similar to those obtained in batch culture.
The beta (1→3) glucanase and protease activities are highest at a dilution rate of about 0.07 h⁻¹ where there is a corresponding peak in yeast lytic activity. The enzyme system has a high beta (1→3) glucanase/protease ratio.

Specific activity (activity/g cells) is calculated using a dry weight per unit optical density correlation of 0.93 g per liter per unit optical density. For this fermentation, beta (1→3) glucanase activity exhibits a peak of specific activity at dilution rates between about 0.05 and about 0.07 h⁻¹. At dilution rates above and below this range the specific activities drop significantly. At dilution rates below 0.07 h⁻¹ catabolite repression is minimal and the rate of glucanase synthesis is dependent mainly upon the concentration of inducer present. Catabolite repression increases with increasing dilution rate until it becomes dominant over induction at dilution rates of about 0.07 h⁻¹ and above. Cytophaga NCIB 9497 has in the past been classified as constitutive for enzyme synthesis. It appears from these continuous culture studies that enzyme synthesis in Cytophaga NCIB 9497 is regulated by induction and catabolite repression.

The specific activity profile for the proteolytic enzyme shows a steady decline with no peak. It does not appear to be inducible but is subject to catabolite repression.

The specific rate of enzyme synthesis (also called the "specific productivity") is the amount of enzyme activity produced per gram of cells per hour. In the range
specific rates of beta (1→3) glucanase and protease synthesis both exhibit peaks. At dilution rates above 0.15 h⁻¹ the rate of glucanase synthesis is low but the rate of protease synthesis shows another peak.

Productivity is activity per liter per hour for enzyme measurement and optical density per liter per hour for measurement of biomass growth. At dilution rates above 0.25 h⁻¹ a peak of high productivity but low concentration occurs with proteolytic and yeast lytic activities. Productivity of beta (1→3) glucanase activity is low at this range of dilution rates. The productivity of biomass increases slowly with increasing dilution rate. Between rates of about 0.03 and about 0.15 h⁻¹, all enzyme activities exhibit a sharp peak in productivity. Productivity of beta (1→3) glucanase activity was 20 times greater and yeast lytic activity 4 times greater than such productivity in batch culture.

Example II

*Gerskovi*a xanthinedy*tica* LL-G109 was grown in continuous culture. The medium contained 2 g/L glucose as the carbon source and 2 g/L yeast wall glucan as the inducer. Enzyme concentration and growth are monitored as well as glucose present in the fermentation broth over a range of dilution rates from 0.03 to 0.38 h⁻¹.

The level of glucose in the fermentation broth was about 0.02 g/L throughout the range of dilution rates used.

The level of beta (1→3) glucanase activity is low when dilution rates are below 0.05 h⁻¹. The level increases
with increasing dilution rate so that activity is high at dilution rates between about 0.05 and about 0.14h⁻¹. Dilution rates above 0.14h⁻¹ result in low activity.

Protease activity is low throughout the range of dilution rates studies, and very low at dilution rates above 0.15h⁻¹.

Specific activity (activity/g cells) is calculated using a dry weight per unit optical density correlation of 0.61 g per liter per unit optical density. For this fermentation, the specific activity of beta (1→3) glucanase is high at dilution rates between about 0.03 and about 0.09h⁻¹, with a peak at a dilution rate of about 0.05h⁻¹. Specific activities at dilution rates below 0.03h⁻¹ and above 0.09h⁻¹ are low. Protease specific activity is greatest at dilution rates below 0.05h⁻¹. Increasing dilution rates result in low protease specific activity.

The specific rate of enzyme synthesis (specific productivity) is the amount of enzyme activity produced per gram of cells per hour. For beta (1→3) glucanase, this value is greatest at dilution rates below 0.15h⁻¹. At dilution rates above 0.15h⁻¹ the specific rate of enzyme synthesis is low. For protease, this value is highest at low dilution rates but diminishes to a low synthesis rate at dilution rates above 0.10h⁻¹.

Productivity is activity per liter per hour for enzyme measurement, and optical density per liter per hour for measurement of biomass growth. Beta (1→3) glucanase activity exhibits high productivity at low dilution rates (below 0.2h⁻¹) with a maximum at about 0.15h⁻¹. Protease productivity is maximized at a dilution rate.
of about 0.15⁻¹ as well. Protease productivity above dilution rate of 0.15h⁻¹ is lower but constant, and below a dilution rate of 0.15h⁻¹ steadily declines.

Example III

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*Oerskovia xanthineolytica* LL-GL09 was grown in continuous culture. The medium contained 8 g/L glucose as the carbon source and 0.5 g/L yeast wall glucan as the inducer. Enzyme concentration and growth are monitored as well as glucose present in the fermentation both over a range of dilution rates for 0.03 to 0.38h⁻¹.

10 The glucose level was close to 0 g/L up to a dilution rate of 0.28h⁻¹, above which it accumulates.

15 The level of beta (1→3) glucanase activity is high at dilution rates between about 0.03 and about 0.2h⁻¹. Activity is low at dilution rates above 0.2h⁻¹.

20 Cell number increased at low dilution rates, dropping drastically above 0.15h⁻¹ and below 0.1h⁻¹.

25 Protease activity is low throughout the range of dilution rates studies, and very low at dilution rates above 0.15h⁻¹.

Specific activity (activity/g cells) is calculated using a dry weight per unit optical density correlation of 0.61 g per liter per unit optical density. For this fermentation, the specific activity of beta (1→3) glucanase is greatest at low dilution rates (below 0.15h⁻¹). For protease this value is highest at dilution rates above 0.25h⁻¹.
Productivity is activity per liter per hour for enzyme measurement, and optical density per liter per hour for measurement of biomass growth. Beta (1→3) glucanase activity exhibits high productivity at dilution rates below 0.2h⁻¹, with a maximum at about 0.15h⁻¹. Protease productivity above a dilution rate of 0.15h⁻¹ is lower but constant, and below a dilution rate of 0.15h⁻¹ steadily declines.
What is Claimed is:

1. A method for preparing a yeast cell lytic enzyme system having desired beta (1\(\rightarrow\)3) glucanase and protease activities which comprises:

   a. inoculating a suitable bacterium which produces the lytic enzyme system onto an appropriate growth medium containing predetermined amounts of a carbon source and an inducer of lytic enzyme activity;

   b. continuously fermenting the bacterium aerobically at an appropriate dilution rate, the dilution rate being varied to obtain the desired protease and beta (1\(\rightarrow\)3) glucanase activities; and

   c. recovering the resulting lytic enzyme system.

2. A method of claim 1, wherein the bacterium is *Cytophaga*, *Oerskovia* or *Arthrobacter*.

3. A method of claim 2, wherein the bacterium is *Oerskovia*.

4. A method of claim 1, wherein the carbon source is glucose.

5. A method of claim 4, wherein the amount of glucose is up to about 20 g/L.

6. A method of claim 1, wherein the inducer is yeast wall glucan.
7. A method of claim 6, wherein the amount of yeast wall glucan used as the inducer is up to about 20 g/L.

8. A method of claim 1, wherein the growth medium is comprised of about 8 g/L glucose and about 0.5 g/L yeast wall glucan.

9. A method of claim 1, wherein the dilution rate provides high beta (1→3) glucanase activity.

10. A method of claim 9, wherein the dilution rate is between about 0.05 and about 0.10 h⁻¹.

11. A method of claim 1, wherein the dilution rate provides high protease activity.

12. A method of claim 11, wherein the dilution rate is between about 0.02 and about 0.10 h⁻¹.

13. A method of claim 1, wherein the dilution rate maintained provides high beta (1→3) glucanase activity and low protease activity.

14. A method of claim 13, wherein the dilution rate is between about 0.15 and about 0.20 h⁻¹.

15. A method of claim 1, wherein the growth medium is comprised of about 2 g/L glucose and about 2 g/L yeast wall glucan.

16. A method of claim 1, wherein the dilution rate maintained provides high beta (1→3) glucanase activity.

17. A method of claim 16, wherein the dilution rate is between about 0.05 and about 0.14 h⁻¹.
18. A method of claim 1, wherein the dilution rate provides high protease activity.

19. A method of claim 18, wherein the dilution rate is between about 0.02 and about 0.14 h⁻¹.

20. A method of claim 1, wherein the dilution rate maintained provides high beta (1→3) glucanase activity and low protease activity.

21. A method of claim 20, wherein the dilution rate is between about 0.15 and about 0.20 h⁻¹.

22. A method of claim 2, wherein the bacterium is Cytophaga.

23. A method of claim 22, wherein the carbon source comprises glucose and yeast extract.

24. A method of claim 23, wherein the amount of glucose used is up to about 20 g/L.

25. A method of claim 23, wherein the amount of yeast extract used is up to about 20 g/L.

26. A method of claim 22, wherein the growth medium is comprised of about 5 g/L glucose and about 10 g/L yeast extract.

27. A method of claim 22, wherein the growth medium pH is about 7.0.

28. A method of claim 22, wherein the growth medium temperature is about 29°C.
29. A method of claim 22, wherein the dilution rate maintained provides high beta (1→3) glucanase activity and high protease activity.

30. A method of claim 29, wherein the dilution rate is between about 0.05 and about 0.10 h\(^{-1}\).

31. A yeast cell lytic enzyme system produced by any of the methods of claims 1, 2, 3, or 22.
**INTERNATIONAL SEARCH REPORT**

**I. CLASSIFICATION OF SUBJECT MATTER** (all several classification symbols apply, indicate all) ²

According to International Patent Classification (IPC) of both National Classification and IPC

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<th>IPC ²:</th>
<th>Cl1N 9/74, Cl1N 9/32</th>
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**II. FIELDS SEARCHED**

Minimum Documentation Searched ⁴

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Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁴

**CHEMICAL ABSTRACT DATA BASE (CAS) 1967-1986; BIOSIS 1969-1986**

**KEY WORD**: PROTEASE, PROTEINASE, GLUCANASE, BACTERIA, CYTOPHAGA, OERSKOVIA, ARTHROBACTER, GLUCAN, YEAST, DILUTE

**III. DOCUMENTS CONSIDERED TO BE RELEVANT**

<table>
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<tr>
<th>Category</th>
<th>Citation of Document, with indication, where appropriate, of the relevant passages ¹⁷</th>
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* Special categories of cited documents: ¹⁵
  - "A" document defining the general state of the art which is not considered to be of particular relevance
  - "E" earlier document but published on or after the international filing date
  - "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
  - "Q" document referring to an oral disclosure, use, exhibition or other means
  - "P" document published prior to the international filing date but later than the priority date claimed

* After document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

* "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

* "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

* "A" document member of the same patent family

**IV. CERTIFICATION**

Date of the Actual Completion of the International Search ⁴ | Date of Mailing of this International Search Report ³ |
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International Searching Authority ¹ | Signature of Authorized Officer ³ |
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<td>Jayne A. Hulett</td>
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Form PCT/ISA/210 (second sheet) (May 1986)
### III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

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<td>Archives of Biochemistry and Biophysics, Volume 145, issued July, 1971 (Baltimore, Maryland, U.S.A.) (K. KITAMURA ET AL), &quot;Lysis of Viable Yeast Cells by Enzymes of Arthrobacter Luteus&quot;, See pages 402-404.</td>
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### FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET


### V. OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE **10**

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. **Claim numbers ............ because they relate to subject matter** not required to be searched by this Authority, namely:

2. **Claim numbers ............ because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out**, specifically:

### VI. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING **11**

This International Searching Authority found multiple inventions in this international application as follows:

1. **As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.**

2. **As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:**

3. **No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:**

4. **As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.**

Remark on Protest
- [ ] The additional search fees were accompanied by applicant's protest.
- [ ] No protest accompanied the payment of additional search fees.